1634

San Diego, October 02, 2003

Dears Ms. Goldberg,

I send you by postmail the responses to your letter postmarked on 07/15/2003 concerning the Application # 09/938,013.

Thank you for reviewing. I could greatly appreciate any assistance you could provide me in order to make this patent application process successful.

Sincerely,

Khue

KHUE VU NGUYEN

P.S.: I sent you the responses by Fax on October 02, 2003.

October 02, 2003

Ms. Jeanine Goldberg
US Patent and Trademark Office
Washington, D.C. 20231

RE: Application # 09/938,013

Dear Ms. Goldberg:

Following our conversation on September 30, 2003, I would like to make the following corrections and clarification:

Specification (4) (page 3)

I made the appropriate correction concerning the passage " in cells ans tissues" on page 1, line 15 as: " in cells and tissues ".

New Matter (5) (page 3)

All of the sequences of the probes 1 (SEQ ID # 1), 2 (SEQ # 2), and 3 (SEQ # 3) are original. Even though, I forgot to include by mistake the sequences of these probes in the application's file on January 7, 2002 !. The sequences of probes 1 and 2 are respectively portions of the whole sequences of the exons 7 and 8 of the SMN gene. The sequences of the exons 7 and 8 of the SMN gene were known at the time the invention was made as you already mentioned. However, the exact sequences of probes 1 and 2 were not taught by any researcher in the instant specification at the time the invention was made.

Claim Rejections-35 USC-112-Scope of Enablement (6) (page 5)

I cancel the claims 2 and 3.

Claim Rejections-35 USC-112-Second Paragraph (7) (page 6)

A- The claims are not limited to these two particular quantification means. BioImager and ELISA with colorimetric detection are only two suggestions for quantification methods.

B- BioImager is referring to any particular device which provide an image of biological material and allows the quantification of the results obtained.

C- I made the appropriate correction as " using labeled nucleotide probes…"

D- The probes 1 (SEQ ID # 1) and 2 (SEQ ID # 2) are respectively portions of the whole

sequences of the exons 7 and 8 of the SMN gene which were determined and published by Lefebvre et al. (GenBank Accession No. U43883) (Ref. # 16 in the application). These probes 1 and 2 were used to detect the presence or absence of exons 7 and 8 respectively for the molecular diagnosis of SMA. The construction of the probes 1 and 2 composes: a/ polymerase chain reaction (PCR) using the primers (g), (h) and (i), (d) for amplifying portions of exons 7 (between base pairs 869-889) and 8 (between base pairs 922-941) respectively. b/ labeling the PCR products so obtained with ^{32}P-dCTP (radioactive label) or biotin 11-dCTP (biotin label, non radioactive label) (see pages 8-9 in the application).

E- The present method developed for measuring specific mRNA of the SMN gene for molecular diagnosis of SMA comprising: a/ obtaining the human samples containing mRNA from control (wild type) and SMA patients. b/ reverse transcription (RT) using the primers (a) and (b) for the synthesis of the first copies of cDNA from the mRNA/SMN and mRNA/HUMEF1AB respectively (the HUMEF1AB gene is used as internal standard for the control of the RT-PCR reactions). c/ polymerase chain reaction (PCR) using the primers (c), (d) and (e), (f) for amplifying in the presence (in use with biotin label and quantifying by means of ELISA method) or absence (in use with radioactive label and quantifying by means of BioImager device) of digoxigenin-dUTP of exons 5-8 (between base pairs 661- 976 of the cDNA/SMN) and between base pairs 672-723 of the cDNA/HUMEF1AB respectively. d/ use of radioactive label for measurement of mRNA: Immobilizing of the PCR products so obtained on the nylon membrane - Hybridizing the immobilized PCR products with radioactive labeled probes 1, 2, and 3 - Detecting the hybridized probe by autoradiography and quantifying by means of BioImager device for diagnosis of SMA. e/ use of biotin label in ELISA method for measurement of mRNA: Hybridizing the PCR products so obtained with biotin labeled probes 1, 2, and 3 - Immobilizing of the hybridized products on the streptavidin precoated plates – Adding the peroxydase-conjugated anti-digoxigenin antibodies - Adding the peroxydase substrate (chromogene and H_2O_2) - Adding H_2SO_4 to stop the reaction - Reading the optical density, OD, by means of a microplate reader for diagnosis of SMA.

F- The claims are not limited to the use of ^{32}P-dCTP and biotin 11-dCTP for labeling. The ^{32}P-dCTP and biotin 11-dCTP are only two suggestions for labeling. Only one label is required: the ^{32}P-dCTP label is for quantifying by BioImager device and the biotin 11-dCTP label is for quantifying by ELISA method.

G- The molecular diagnosis of SMA refers to the diagnostic method based on the detection of SMA at the DNA or RNA levels. In the present invention, the detection of SMA is at the mRNA level.

H- I cancel the claims 2 and 3.

Claim Rejections-35 USC-103 (9) (pages 9-12)

The gene most highly associated with SMA is the survival motor neuron (SMN) gene, located in the chromosome 5q13.3. The SMN gene is composed of 8 exons extending over 20 kb and has telomeric (SMNT) and centromeric(SMNC) copies that are highly homologous. The SMNT and its centromeric copy (SMNC) differ in their exons by only two base pairs, one in

exon 7 and one in exon 8. This difference allows the distinction of the SMNT gene from SMNC by single-strain conformation polymorphism (SSCP) analysis (Lefebvre et al., Cell, 1995, **80**, 155-165) or by use of restriction enzymes (Van Der Steege et al., Lancet, 1995, **345**, 985-986).. Only homozygous absence of SMNT is responsible for SMA, while homozygous absence of SMNC, found in 5% of control, has no-clinical phenotype. It was reported that 93% of all types of SMA patients carry homozygous deletions of exons 7 and 8 or only exon 7, of the SMNT gene, either because of conversion of sequences in the SMNT gene to those in the SMNC gene or as a results of SMNT gene deletions (Lefebvre et al., Cell, 1995, **80**, 155-165; Burghes et al., American Journal of Human Genetics, 1997, **61**, 9-15; Campbell et al., American Journal of Human Genetics, 1997, **61**, 40-50; DiDonato et al., Annals of Neurology, 1997, **41**, 230-237). In rare cases, small mutations in SMNT gene without a deleted or sequence-converted SMNT gene, are responsible for the disease (Lefebvre et al., Cell, 1995, **80**, 155-165; Bussaglia et al., Nature Genetics, 1995, **11**, 335-337; Brahe et al., Human Molecular Genetics, 1996, **5**, 1971-1976; Parsons et al., Human Molecular Genetics, 1996, **5**, 1727-1732). The identification of small mutations in SMNT gene is complicated by the presence of SMNC gene. The absence of detectable SMNT exons 7 and 8 in SMA patients by means of the SSCP technique or restriction enzymes, is being utilized as a powerful diagnostic test for SMA. However, these methods for molecular diagnosis of SMA at the DNA level are not quantitative and they can not detect individuals with heterozygous deletions of SMNT gene (SMA carriers) and distinguish between a non-5q SMA patient (in whom SMNT is absent on one chromosome and an unknown alteration in the SMNT gene is present on the other chromosome). To allow identification of SMA carriers, quantitative polymerase reaction (PCR) methods to determine the SMNT and SMNC gene-copy number have been reported (McAndrew et al., American Journal of Human Genetics, 1997, **60**, 1411-1422; Chen et al., American Journal of Medical Genetics, 1999, **85**, 463-469; Ogino et al., Journal of Molecular Diagnostics, 2001, **3**, 150-157). The quantitative PCR assay to measure SMNT and SMNC gene-copy number provides clear advantages over existing qualitative methods. However, to avoid inaccuracies in the quantification of copy number, the quantitative PCR assay requires technical precautions such as the controls prepared by the same extraction method, the preflashing the film and monitoring exposure times to ensure the linearity of film response for autoradiography. Moreover, the direct testing of heterozygous at the DNA level by the quantitative PCR assay is compromised by the presence of two SMNT copies per chromosome in about 4% of individuals. Thus, the development of another approach for accurate quantitative molecular diagnosis of SMA is needed. For such a purpose, we developed a quantitative method for molecular diagnosis of SMA at the mRNA level. Indeed, it was reported that the SMNT transcripts were absent and that the SMNC transcripts were solely present in the patients lacking the SMNT gene on both mutant chromosomes, while control individuals expressed both RNA transcripts. The SMNC transcripts, but not the SMNT transcripts, may undergo alternative splicing of exon 7 to produce transcripts lacking this exon. Analysis of a control population showed that the SMNT gene was present in all individuals while the SMNC gene was present in 95% (Lefebvre et al., Cell,1995, **80**, 155-165). Taking into account the about mentioned problem, the quantitative method based on the measurement of specific messenger RNA (mRNA) may be useful for the quantitative molecular diagnosis of SMA. The results obtained by measuring the amount of cytosolic mRNA from human muscle cells demonstrate that the measurement of specific mRNA can be used as a quantitative method for the molecular diagnosis of SMA. A recent research work performed by Jong et al., Journal of the Neurological Sciences, 2000, **173**, 147-153, showed that the measurement of mRNA

transcripts of SMN gene can be used as a novel diagnostic method. These authors, in a semi-quantitative method using Image Analysis System, analyzed the mRNA transcripts of the SMN gene in the peripheral blood mononuclear cells of normals, carriers and SMA patients. They calculated the ratio between RT-PCR products containing exon 7 and lacking exon 7, and the ratio between RT-PCR products containing exon 7 and β-actin from normal subjects, SMA patients and carriers. A significant difference in ratios was found and they suggested that analyzing the mRNA expression of SMN gene offers an apparently reliable technique for separating SMA patients, carriers, and normal individuals.

In conclusion, the originality of the present invention resides:

1. on the use of nucleotide probes 1, 2, and 3 which were originals (their exact sequences were not taught by any researcher in the instant specification at the time the invention was made). These probes 1, 2, and 3 are used in the hybridization procedure with the RT-PCR products for the quantitative molecular diagnosis of SMA.

2. on the use of messenger RNA (mRNA) for the quantitative molecular diagnosis of SMA.

The results of the present invention were accepted for publication in Analytical Letters, 2002, **35**, 1135-1148.

I hope I have adequately responded to your questions and I would like to make the corrections of the Claiming paragraph as follows:

VI - CLAIMING

What is claimed is:

A quantitative method using labeled nucleotide probes 1, 2, and 3 for measurement of specific mRNA for molecular diagnosis of SMA, comprising:

(1) obtaining the human samples containing mRNA from control (wild type) and SMA patients.

(2) reverse transcription (RT) using the primers (a) and (b) for the synthesis of the first copies of cDNA from the mRNA/SMN and mRNA/HUMEF1AB respectively (the HUMEF1AB gene is used as internal standard for the control of the RT-PCR reactions).

(3) polymerase chain reaction (PCR) using the primers (c), (d) and (e), (f) for amplifying in the presence (in use with biotin label and quantifying by means of ELISA method) or absence (in use with radioactive label and quantifying by means of BioImager device) of digoxigenin-11-dUTP.

(4) use of radioactive label for measurement of mRNA: Immobilizing of the PCR products so obtained on the nylon membrane – hybridizing the immobilized PCR products with radioactive ^{32}P-dCTP labeled probes 1, 2 , and 3 – Detecting the hybridized probe by autoradiography and quantifying by means of BioImager device for diagnosis of SMA.

(5) use of biotin label in ELISA procedure for measurement of mRNA: Hybridizing the PCR products so obtained with biotin-11-dCTP labeled probes 1, 2, and 3 – Immobilizing of the hybridization products on streptavidin coated plates – Adding the peroxydase-conjugated anti-digoxigenin antibodies – Adding the peroxidase substrate (chromogene and H_2O_2) – Adding H_2SO_4 to stop the reaction – Reading the optical density, OD, by means of microplate reader for diagnosis of SMA.

Thank you for reviewing my application; I would greatly appreciate any assistance you could provide me in order to make this patent application process successful. If you have further questions, please contact me at (619) 543-3623; Fax: (619) 543-7868; e-mail: kv52nguyen@yahoo.com

Sincerely,

Khue Vu Nguyen, Ph.D.
2828 University Avenue, Apt # 303
San Diego, CA 92104

Encl: Photocopy of the article of Jong et al., Journal of the Neurological Sciences, 2000, **173**, 147-153.
Photocopy of our article: Nguyen et al., Analytical Letters, 2003, **35**, 1135-1148. (published the results of the present invention).

Journal of the Neurological Sciences 173 (2000) 147–153

Journal of the Neurological Sciences

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Analysis of the mRNA transcripts of the survival motor neuron (*SMN*) gene in the tissue of an SMA fetus and the peripheral blood mononuclear cells of normals, carriers and SMA patients

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Abstract

Spinal muscular atrophy (SMA) is a disorder characterized by degeneration of the anterior horn cells of the spinal cord. The gene most highly associated with SMA is the survival motor neuron (*SMN*) gene. In this study, we present an analysis of messenger RNA (mRNA) expression of the *SMN* gene in peripheral blood mononuclear cells in normal subjects, SMA carriers and patients from 20 SMA families. We found at least 6–8 different transcripts of *SMN* gene formed by alternative splicing involving exons 3, 5 and 7. We compared the transcripts from the different types of SMA and found no definite differences in transcript patterns and amounts. Normal subjects with the telomeric *SMN* (SMN^T) gene only had variable splicing resulting in several transcripts, the most dominant being a transcript containing all coding regions. However, SMA patients with the centromeric *SMN* (SMN^C) gene only had a higher degree of splice variation and tended to show little or no exon 7. These results demonstrate that SMN^T and SMN^C genes participate in alternative splicing phenomena. The different splicing patterns support the view that the SMN^T gene is responsible for SMA disease. We also analyzed the transcripts from several tissues of an SMA fetus who had a homozygous SMN^T gene deletion. Different splicing patterns were also found in these tissues, and were similar to the splicing pattern of leukocytes. We compared the major transcripts from exons 4 to 8 of both the SMN^T and SMN^C genes and found that the relative proportion varied among normal subjects, SMA carriers and patients. This approach could be used as a novel diagnostic method. We suggest that analyzing the mRNA expression of the *SMN* gene in peripheral blood mononuclear cells offers an apparently reliable technique for separating SMA patients, carriers, and normal individuals.

Keywords: Spinal muscular atrophy (SMA); SMA carrier; Survival motor neuron (*SMN*) gene; messenger RNA (mRNA); Transcript; Alternative splicing

1. Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive disorder characterized by degeneration of the anterior horn cells in the spinal cord, and sometimes also in the brainstem nuclei, leading to symmetric weakness and wasting of voluntary muscles. Three different forms of childhood SMA have been recognized on the basis of age of onset and progression of the disease: Werdnig-Hoffmann disease (type I), Dubowitz disease (type II), and Kugelberg-Welander disease (type III) [1–8]. All three forms are linked to chromosome 5q13, in a particularly

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PII: S0022-510X(99)00325-1

unstable region prone to large-scale deletions [9–13]. The characterization of these deletions has led to the identification of neuronal apoptosis inhibitory protein (*NAIP*) and survival motor neuron (*SMN*) genes associated with SMA [14,15]. The *NAIP* and the highly homologous gene XS2G3 have been found to be more frequently deleted in type I SMA (45%) than in the type II and III SMA (18%) [15,16]. The *SMN* gene is composed of eight exons and has telomeric (SMN^T) and centromeric (SMN^C) copies that are highly homologous, displaying only in eight base pair (bp) differences within their 3′ ends. Two of these bp exchanges, in exons 7 and 8, allow us to distinguish SMN^T from SMN^C on DNA and RNA levels [14]. More than 95% of all types of SMA patients carry homozygous deletions of exons 7 and/or 8 of the SMN^T gene, but there is no definite correlation between the extent of the apparent deletion and the clinical severity [14,17–24]. In this paper, we present an analysis of messenger RNA (mRNA) expression of the *SMN* gene in peripheral mononuclear blood cells among normal subjects, SMA patients and carriers, and the tissues of a fetus who had a homozygous SMN^T gene deletion, to evaluate differences in *SMN* gene expression.

2. Materials and methods

2.1. Subjects and blood samples

We examined blood samples from 30 unrelated normal subjects, 20 SMA patients, and their 39 asymptomatic carrier parents (one of the parents is dead), and several tissues from an SMA fetus that were collected after abortion from a family with type I SMA. Of these SMA patients, 5 from 5 families belonged to type I, 6 from 6 families to type II, and 9 from 9 families to type III. All patients fulfilled the diagnostic criteria of SMA as defined by the International SMA Consortium [25]. The 20 SMA patients and an SMA fetus all had homozygous deletion of SMN^T gene but carried SMN^C gene, and changes in their *SMN* and *NAIP* genes have previously been reported [19,26].

2.2. RNA isolation for reverse transcriptase-polymerase chain reaction (RT-PCR) and sequencing analysis

Total RNA was isolated using a commercial kit (TRI$_{ZOL}^{®}$ Reagent, Life Technologies, Inc., Grand Island, NY, USA). First-strand synthesis from total RNA was carried out using a random primer 5′-TNNNNNNNNNN-3′ and MMLV reverse transcriptase (Promega, Madison, WI, USA). The single stranded cDNA was PCR-amplified using two pairs of primers to cover all *SMN* gene coding regions of the cDNA. The first pair of primers was amplified from the 5′ non-coding region to exon 4 near exon 5, with the forward primer (P1): 5′-CTGCGCATCCGCGGGTTTGCTATGGC-3′ (cDNA nt 13 to nt 38), and the reverse primer (P2): 5′-TCCCAGTCTTGGCCCTGGCAT-3′ (cDNA nt 651 to nt 631). The second pair of primers was amplified from the end of exon 4 to exon 8, with the forward primer (P3): 5′-ATGCCAGGGCCAAGACTGGGA-3′ (cDNA nt 631 to nt 651), and the reverse primer (P4): 5′-ATTCCAGATCTGTCTGATCG-3′ (cDNA nt 985 to nt 966). To amplify the full length of the cDNA, we used primers P1 and P4.

The PCR reaction was performed as previously described [19]. The amplification conditions were as follows: denaturation at 94°C for 2 min, annealing at 65°C for the first pair (P1 and P2), at 56°C for the second pair (P3 and P4), at 56°C for primers P1 and P4, and extension at 72°C for 3 min, for a total of 35 cycles (55 cycles for primers P1 and P4). The PCR products were electrophoresed and analyzed using 3% agarose gels. For isolation of fragments from PCR products, the products were electrophoresed in 3% low melting agarose gels, and the fragments were excised after separation. They were then purified using a commercial kit (Qiaex II gel extraction kit, QIAGEN Inc., Chatsworth, CA, USA), and the purified fragments were sequenced by the cycling sequencing method (AmpliCycle™ Sequencing kit, Roche Molecular Systems, Inc., Branchburg, NJ, USA).

2.3. Southern blot analysis of RT-PCR products of the whole or parts of SMN gene

Southern blot analysis was performed according to a standard method [27], using the biotin-labeled probes (Biotin-5′-GTCCCGGAGCAGGAGGATTCCGTGCTG-TTC-3′ located at cDNA nt 67 to nt 96 of exon 1, Biotin-5′-CCAGGTCTAAAATTCAATGGCCCA-3′ located at cDNA nt 661 to nt 684 of exon 5), and the result was detected by Luminescent Detection Kit (Boehringer, Mannheim GmbH, Germany).

2.4. Quantitation of the RT-PCR products of normal subjects, SMA patients and carriers

Two methods were used to semi-quantitatively analyze the numbers of transcripts containing or lacking exon 7 [28]. The first method was used to analyze the ratio between the RT-PCR products containing exon 7 and lacking exon 7 of normals subjects, SMA patients and carriers, by using the above-mentioned primers 3 and 4. The second method used upstream primer (P5): 5′-CTCCCATATGTCCAGATTCTCTTG-3′ (from part of codon 247 to part of codon 255) and downstream primer (P6): 5′-ACTGCCTCACCACCGTGCTGG-3′ (cDNA nt 1191 to nt 1171), for RT-PCR to amplify part of exon 6, all of exon 7 and part of exon 8, and a pair of β-actin primers (upstream primer: 5′-TACGCCAACACAGTGCT-GTCTG-3′ and downstream primer: 5′-CTGCTTGCTGAT-

CCACATCTGC-3′) were also used simultaneously to amplify the transcripts of β-actin in the same reaction tube as an internal control to quantify the amount of RNA in the reaction tubes. The second method also analyzed the ratio between RT-PCR products containing exon 7 and lacking exon 7, and the ratio between RT-PCR products containing exon 7 and β-actin.

The intensity of the RT-PCR products containing exon 7 or lacking exon 7, or β-actin were scanned and analyzed with the Collage™ Image Analysis System (Fotodyne Incorporated, Hartland, WI, USA) to calculate the ratio of these products. Analysis of variance (ANOVA) followed by Neuman-Keuls test was used for statistical analysis of any significant differences [19].

3. Results

3.1. mRNA transcripts of SMN^T and SMN^C genes

We specifically analyzed the *SMN* gene transcripts of normal subjects who had only SMN^T and of SMA patients who had only SMN^C. The results are shown in Fig. 1A. Using primers 1 and 4 in all cases, we found transcripts consisting of 973 bp, 919 bp, 877 bp, 823 bp, 772 bp, 718 bp, 676 bp, and 622 bp. After purifying and sequencing these eight fragments, we found that the 973 bp fragment contained all of the cDNA sequences of *SMN* gene, and the 919 bp fragment had no exon 7 sequence. The 877 bp fragment and the 823 bp fragment did not carry exon 5 sequence, and the latter also lacked exon 7. The 772 bp fragment did not contain exon 3 sequence, and the 718 bp fragment was lacking both exons 3 and 7 sequences. The 676 bp fragment contained no sequences of exons 3 and 5, and the 622 bp fragment contained no sequences of exons 3, 5 and 7 (Fig. 1B). Most of the major transcript of SMN^T gene (973 bp) contained all exons. However, there were also minor transcripts lacking exon 7 (919 bp) or 5 (877 bp), and a few transcripts lacking either exon 3 (772 bp), both exons 5 and 7 (823 bp), or exons 3 and 7 (718 bp) (Fig. 1A, lane 7). For SMN^C gene, the major transcript (919 bp) lacked exon 7, followed by minor transcripts lacking exons 3 and 7 (718 bp), exons 5 and 7 (823 bp), and exons 3, 5 and 7 (622 bp), accompanied by lesser numbers of transcripts containing either all exons (973 bp), or lacking exon 3 (772 bp) (Fig. 1A, lanes 1 to 3). SMA carriers (Fig. 1A, lanes 4, 5) and normal subjects (Fig. 1A, lane 6) sharing both SMN^T and SMN^C genes also had similar transcripts. SMN^T gene yielded more exon 7-containing transcripts than did SMN^C gene (Fig. 1B). We compared transcripts from the different types of SMA and found no definite differences in transcript patterns and amounts. We also could not find a correlation between the genotype and the mRNA amounts or patterns in all types of SMA patients.

3.2. Quantitative analysis of the RT-PCR products of normal subjects, SMA patients and carriers

For more efficient amplification of the *SMN* gene, we used two sets of primers to amplify parts of the gene. Two PCR products were found (639 bp and 438 bp) after amplification of the cDNA sequence nt 13 to nt 651 using primers 1 and 2 (Fig. 2A). Four fragments (355, 301, 259 and 205 bp) were produced from the amplification using primers 3 and 4, covering the cDNA sequence from nt 631 to nt 985 (Fig. 2B). To determine the significance of these fragments, we directly sequenced all six fragments. We found that the 639 bp and 355 bp fragments were normal transcripts containing all exons of the amplified region.

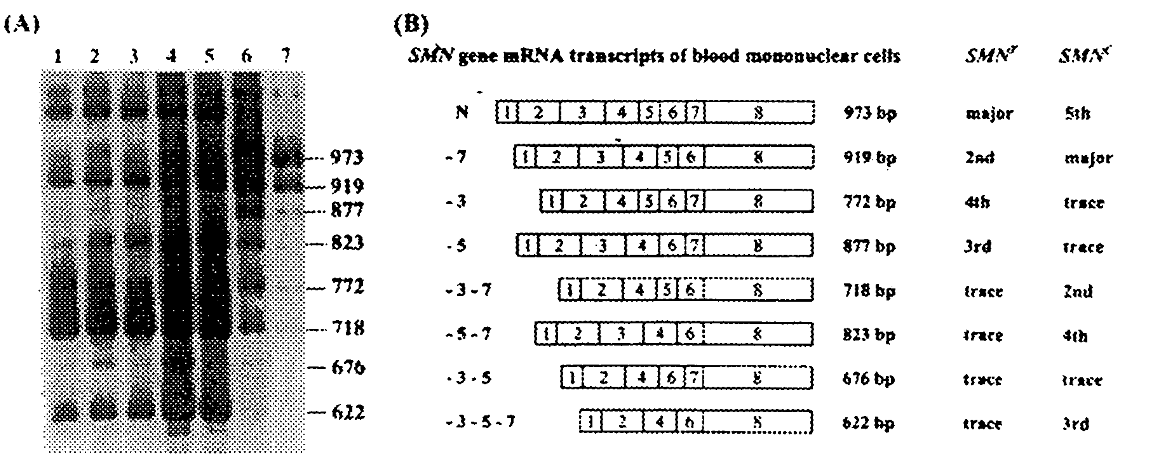

Fig. 1. (A) Southern blot analysis of RT-PCR products of *SMN* gene transcripts. Lanes 1, 2 and 3 are from type I, II and III SMA patients, respectively. Lanes 4 and 5 are SMA carriers. Lane 6 is from a normal subject who has SMN^C and SMN^T genes; and lane 7 is from a normal subject who has the SMN^T gene only. (B) Summary of different types of SMN gene transcripts. There are eight transcripts of various combinations of deletion in exons 3, 5 and 7. The number of different transcripts of SMN^C and SMN^T genes vary, and the most abundant one of the SMN^T gene contains all exons. For SMN^C gene, the major transcript contains little or no exon 7.

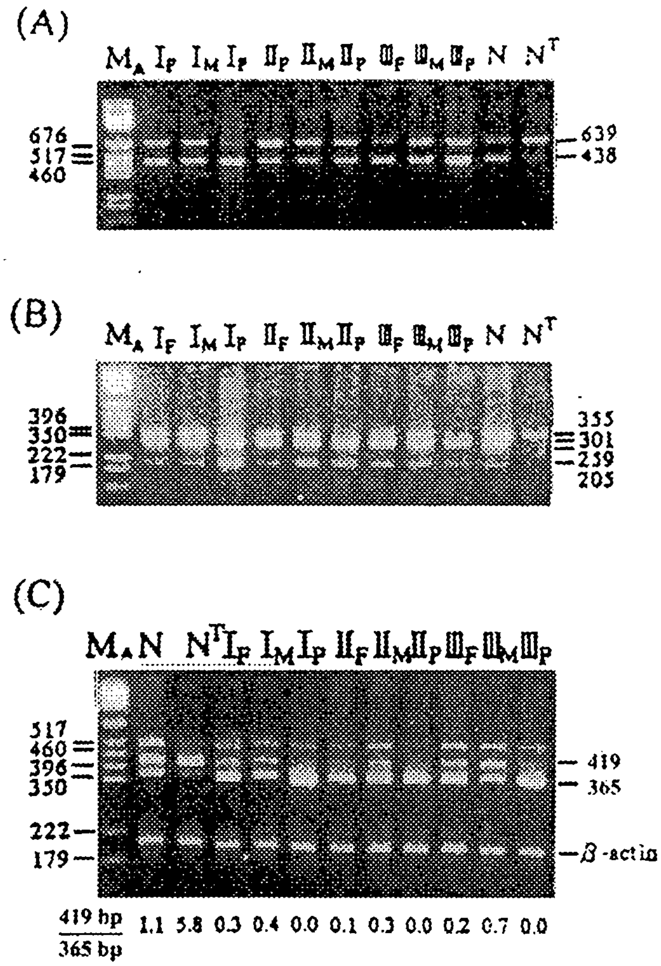

Fig. 2. Results of RT-PCR of *SMN* gene transcripts from peripheral blood mononuclear cells in normal subjects, different types of SMA patients and carriers. (A) RT-PCR of exon 1 to exon 4. (B) RT-PCR of exon 4 to 8. (C) RT-PCR of part of exon 6 to 8 with β-actin as an internal control. M_A: marker; I, II, III: SMA type, F: father, M: mother, P: proband, N: normal with both SMN^T and SMN^C genes. N^T: normal with SMN^T only.

The 438 bp fragment lacked exon 3, the 301 bp fragment lacked exon 7, the 259 bp fragment lacked exon 5, and the 205 bp fragment lacked both exons 5 and 7. We found the only difference among SMA patients, carriers and normal subjects was different yields of the fragments, especially the transcripts which contained or lacked exon 7. We scanned the ratio between the exon 7-containing fragment (355 bp) and exon 7-deficient fragment (301 bp), in each case using densitomeric analysis [19]. The ratios of SMA patients were near zero, near one for carriers, but greater than one for the normal subjects (Fig. 3). To further confirm the results, we multiplexly amplified the β-actin and *SMN* genes (primers 5 and 6) in the same reaction as an internal control, and then analyzed the ratio between exon 7-containing product (419 bp) and exon 7-deficient product (365 bp) of the *SMN* gene (Fig. 2C). This process yielded the same results as the previous method (Fig. 3).

3.3. mRNA transcripts from different tissues of an SMA fetus

The results of RT-PCR analysis of *SMN* genes from different tissues are shown in Fig. 4A. All of the tissues from the SMA fetus had similar transcripts, but they differed from the transcripts of the peripheral blood mononuclear cells of SMA patients, and normals. The transcripts of fetal tissue had much less alternative splicing in comparison with SMA patients (Fig. 4B).

4. Discussion

The majority of SMA patients lack the SMN^T gene, corroborating the hypothesis that it is an SMA-determining gene [14,17–20,23,24,29,30]. Among the transcripts from

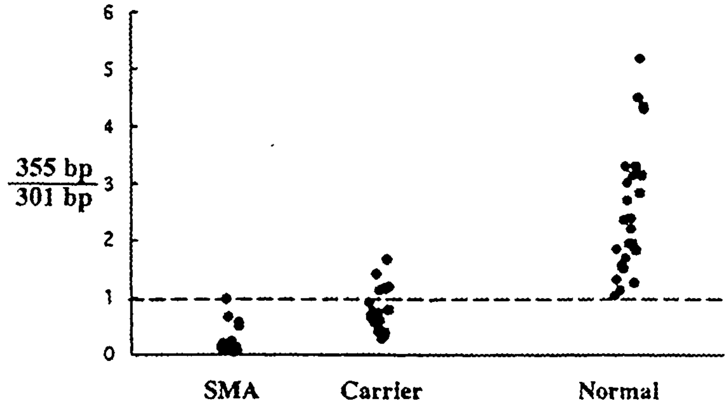

Fig. 3. The exon 7-containing fragment (355 bp)/exon 7-deficient fragment (301 bp) ratios vary among SMA patients, carriers and normal subjects in densitometer analysis ($P<0.0001$).

SMN^T and SMN^C genes, we found alternative splicing phenomena, involving one exon or combinations of exons 3, 5 and 7. The majority of transcripts from the SMN^T gene are full length, incorporating exon 7. However, major transcripts of the SMN^C gene have a high degree of alternative splicing and tend to show little or no exon 7. These results confirm that these two genes can be distinguished at the RNA level, and also the importance of exon 7 in maintaining the function of the *SMN* gene [14]. Its gene product, the *SMN* protein, is directly involved in the biogenesis of spliceosomal small nuclear ribonucleoproteins (snRNP). Defects in spliceosomal snRNP biogenesis, may be the cause of SMA [31,32].

Exon 7-containing transcripts were less detectable in SMA patients, no matter which type of SMA they had. These results differ from a study of *SMN* protein levels which showed quantitative differences among the three types of SMA [33]. The differences may be because the exon 7-containing transcripts are too rare to be detected accurately. In this study, we found that all three types of SMA patients had abundant exon 7-deficient mRNA transcripts. If these transcripts were translated normally, theoretically the protein should be detectable by antibody, as in the study by Lefebvre et al. [33]. However, using their data, we were unable to observe this phenomenon. We therefore suggest that *SMN* transcripts lacking exon 7 may

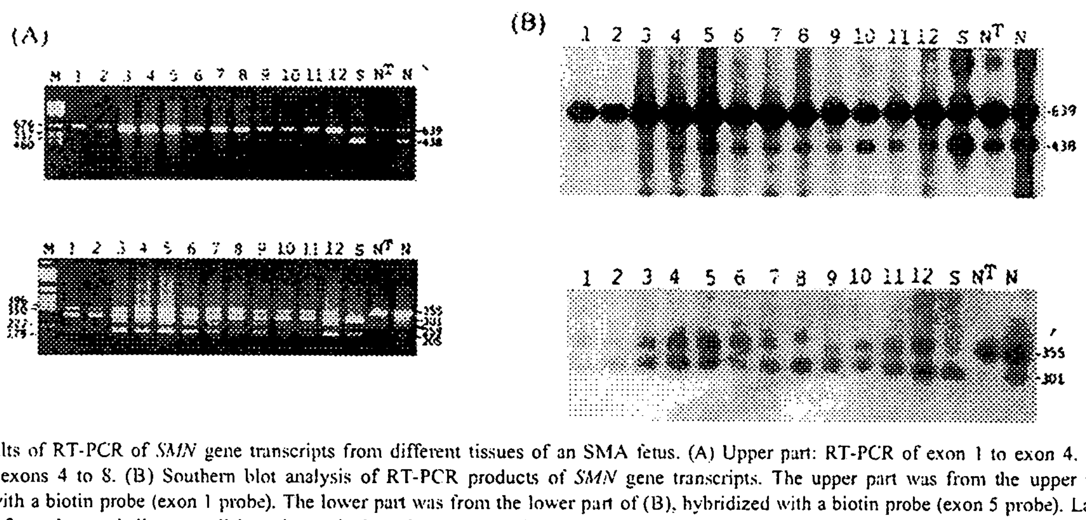

Fig. 4. Results of RT-PCR of *SMN* gene transcripts from different tissues of an SMA fetus. (A) Upper part: RT-PCR of exon 1 to exon 4. Lower part: RT-PCR of exons 4 to 8. (B) Southern blot analysis of RT-PCR products of *SMN* gene transcripts. The upper part was from the upper part of (A), hybridized with a biotin probe (exon 1 probe). The lower part was from the lower part of (B), hybridized with a biotin probe (exon 5 probe). Lanes 1 to 12 were tissues from the cerebellum, small intestine, spinal cord, temporal lobe of the brain, kidney, brainstem, lung, liver, frontal lobe of the brain, heart, biceps brachii muscle, and occipital lobe of the brain, respectively. M: marker, S: type I SMA patient. N: normal with both SMN^T and SMN^C genes. N^T: normal with the SMN^T gene only.

undergo RNA metabolism by a different pathway, or exist in a unstable state, which leads to no or minimal *SMN* protein production. This lack of exon 7 coding amino acids may result in the loss of an important function and thus cause the disease. This proposal needs further study.

Both SMN^T and SMN^C are transcribed in a variety of human tissues, including brain, heart, kidney, liver, intestine, lung, skeletal muscle, and spinal cord. We compared the transcripts of different tissues of an SMA fetus, and found that all the tissues had alternative splicing of exons 3, 5 and 7, which were similar with the transcripts of peripheral mononuclear cells of SMA patients. The muscle tissue also had alternative splicing phenomenon, which involved exons 3, 5 and 7. This result was slightly different from the study of Gennarelli et al. [34] in which found that only exons 5 and 7 had alternative splicing. We also compared the amount of *SMN* gene transcripts between SMA fetal tissue and peripheral blood of SMA patients. We found that there were differences between the transcripts containing exon 7 and transcripts lacking exon 7. The fetal tissue had more transcripts containing exon 7. These results may explain why SMA patients develop symptoms after birth. The SMN^C gene produced a variety of different isoforms, including transcripts containing little or no exon 7, as well as lesser amount of full length *SMN* transcripts [14,34]. The phenomenon may also provide the basis for SMA therapy in the future, namely by modulation of SMN^C to produce more mRNA which contains exon 7. Meanwhile, the universal alternative splicing expression of *SMN* gene may have been suggested by the results of research on peripheral mononuclear cells.

When we analyzed mRNA expression of the *SMN* gene in peripheral blood mononuclear cells, we found that normal subjects had the highest number of transcripts containing exon 7, SMA patients the lowest, and carriers were in between. These findings may provide a novel method for carrier detection, even in non-deletional cases. Although there was a strong correlation of the ratio between transcripts containing exon 7 and transcripts lacking exon 7 among normal subjects, SMA patients and carriers, there is also a significant percentage of the population with SMN^Tonly (5% for Chinese and Caucasians). This allele will influence the results of carrier detection when using the RT-PCR method described in this study.

In summary, the PCR based genomic DNA assay can easily detect most of the SMA patients who carry a partially deleted SMN^T gene. However, this method can not be used to detect non-deletional mutations, such as point mutation, and to identify SMA carriers. mRNA expression of the *SMN* gene in peripheral blood mononuclear cells was designed for screening nondeleted mutations of SMA patients, and provided an apparently reliable technique for separating SMA patients, carriers and normal individuals.

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UTILIZATION OF NUCLEOTIDE PROBES FOR THE MEASUREMENT OF SPECIFIC MESSENGER RNA: APPLICATION FOR MOLECULAR DIAGNOSIS OF AUTOSOMAL RECESSIVE SPINAL MUSCULAR ATROPHY

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ABSTRACT

Spinal muscular atrophy (SMA) is a lethal autosomal recessive disease. SMA is characterized by degeneration of motor neurons in the spinal cord, causing progressive weakness of the

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limbs and trunk, followed by muscle atrophy. The gene most highly associated with SMA is the survival motor neuron (SMN) gene. This paper describes the results concerning the development of a quantitative method for the molecular diagnosis of SMA by measuring the amount of cytosolic mRNA from human muscle cells. The procedures using radioactive material and the Enzyme-Linked Immunosorbent Assay (ELISA) non-radioactive method were developed using ^{32}P-dCTP labeled and biotinylated nucleotide probes, respectively; the results obtained demonstrate that the measurement of specific mRNA could be used as a quantitative method for the molecular diagnosis of SMA. There was a perfect concordance of the results obtained between the procedure using radioactive material, the ELISA method and the single-strand conformation polymorphism (SSCP) analysis regarding negative and positive SMA samples. All values obtained for the control group were significantly greater than the ones obtained for the SMA positive samples (33–76% in radioactive method and 38–54% in ELISA method). The methods developed in this study may be applicable to the diagnosis (detection of homozygous and heterozygous deletions in exons 7 and 8 of the SMN gene) and the control of mRNA concentrations in future gene therapy of patients with SMA.

Key Words: Nucleotide probes; Molecular diagnosis; Autosomal recessive spinal muscular atrophy; Radioactive label; Biotin label; Enzyme-Linked Immunosorbent Assay

INTRODUCTION

New gene sequences are discovered daily, and advanced molecular biological techniques are revolutionizing clinical practices in genetic disorders, oncology, infections diseases, etc. Although the current major focus is on using DNA to identify disease genes, mutations, and translocations, or foreign genes as infectious agents, the quantification of various specific messenger RNA (mRNA) molecules in cells and tissues is an attractive field in diagnostic molecular pathology because the concentrations of each specific mRNA are different in normal and disease states. These concentrations also change rapidly in response to various clinical treatments. Among the

technologies for assay of mRNA are Northern blotting,[1] RNase protection assay,[2] reverse transcription followed by polymerase chain reaction (RT-PCR),[3,4] in situ hybridization[5] and in situ PCR.[6] Currently, a variety of detection labels can be used, e.g., radioisotopes, fluorescence,[7] and chemiluminescence.[8] Furthermore, once mRNA is reverse-transcribed into cDNA, various gene amplification techniques[9–11] may be applicable. However, because each assay has its own problems, no assay has been accepted as routine for molecular diagnostic purposes, and none of these assays allow researchers to use conventional colorimetric Enzyme-Linked Immunosorbent Assay (ELISA) readers, which are widely available in any laboratory.

To address the problem described, the important task is to identify a procedure to measure specific mRNA for the molecular diagnosis of genetic disorders. Among a variety of genetics disorders, spinal muscular atrophy (SMA) is a lethal autosomal recessive disease affecting 1 in 6,000 newborns, and is one of the most common genetic causes of death in childhood.[12–14] SMA is characterized by the degeneration of motorneurons from the ventral horns of the spinal cord, leading to symmetrical paralysis of voluntary muscles with muscular atrophy. Three different clinical syndromes of SMA (SMA types I, II, and III) can be defined on the basis of age of onset, milestones of development, and age of survival.[15]

All three types of SMA map to chromosome 5q13.3. Recently, Lefebvre et al.[16] identified the SMN gene (Survival Motor Neuron, T_{BCD541}) with 8 exons extending over approximately 20 kb. There is a high homologous copy of the SMN gene in the centromeric repeating unit (C_{BCD541}); this copy is present in 95.5% of control and hampers detection of absence of the SMN gene. The SMN gene and its centromeric copy differ in their exons by only two base pairs, one in exon 7 and one in exon 8; this difference thus allows the distinction of the SMN gene from its centromeric copy by single-strand conformation polymorphism (SSCP) analysis,[16] or by the use of restriction enzymes.[17] The SMN gene was either absent or interrupted in exons 7 and 8 in the majority of patients (98%), for all three types of SMA.[16]

The qualitative techniques for molecular diagnosis of SMA at the DNA level using the SSCP technique[16] and restriction enzymes[17] have actually become feasible by looking at the presence or absence of exons 7 and 8 of the SMN gene on chromosome 5q13.3. In an attempt to develop a quantitative method for the molecular diagnosis of SMA, we use the labeled nucleotide probes (labeling with ^{32}P-dCTP and with biotin) in both the procedure using radioactive material and the Enzyme-Linked Immunosorbent Assay (ELISA) nonradioactive method for the measurement of specific mRNA. Both exons 7 and 8 of the SMN gene

are checked for the diagnosis. The sample used for analysis can be either a biological fluid such as whole blood, or a fraction of cells or tissue, in which the RNA can be isolated. In the study, as described herein, procedures which utilize human muscle cells from muscle biopsies for analysis are used.

MATERIALS AND METHODS

Cell Culture

The methodologies of sample taking (fractions of human muscles from donors suffering from SMA and from corresponding normal controls), culture and maintenance of human muscle cells were established according to the techniques described by Askanas and Engel[18] and Askanas and Gallez-Hawkins.[19]

Isolation of RNA

The ribonucleic acid (RNA) was isolated from the cells according to the method described by Sambrook[1] using guanidine/phenol (*Tris* Reagent™, Euromedex, 67460 Souffelweyersheim, France). The RNA was dissolved in water pre-treated by 0.1% (v/v) diethyl pyrocarbonate (DEPC, Sigma, St. Louis, MO). This RNA solution is ready for subsequent treatment for synthesis of the cDNA. The purity and integrity of the RNA used were analyzed by electrophoresis on agarose gel in denaturing conditions.[1]

Reverse Transcription

The synthesis of the cDNA was performed by reverse transcription (RT).[1] The first copies of cDNA were synthesized using two oligonucleotides (a) and (b) (Genosys Biotechnologies, Europe, Ltd., France) generated with the following sequences: 5′CACATTGCATTTG3′ (a) and 5′CTGTCTGTCTCA3′ (b). These oligonucleotides (a) and (b) were selected taking the complementary sequence to allow RT. The oligonucleotide (a) was based on the SMN sequence described by Lefebvre et al.[16] between base pairs 1097 and 1109. The oligonucleotide (b) was based on the sequence of the HUMEF1AB gene, encoding for the human elongation factor 1-alpha (EF1A), described by Ann et al.[20] between base pairs 881 and 892. This HUMEF1AB gene was used as internal standard for the control of the RT-

PCR reactions. The M-MLV Reverse Transcriptase enzyme (Gibco BRL®, Life Technologies Sarl, BP 96, 95613 Cergy Pontoise, France) was used for the reverse transcription reaction. This reaction was effected as follow:

To 1.5 µg of total RNA were added 0.3 nmol of oligonucleotides each, 0.6 nmol of 1,4-dithiothreitol threo-1,4-dimercapto-2,3 butanediol, DTT (Gibco BRL®), and nucleotides dATP, dCTP, dGTP, dTTP at a concentration of 6×10^{-5} mol L^{-1}. The reaction was conducted in the presence of a reaction buffer for RT of the Gibco BRL® kit and in a total volume of 60 µL. After heating the mixture to 90°C for 2 min and then cooling it on ice for 1 min, 200 U M-MLV were added; and the mixture was left to 25°C for 10 min and then at 42°C for 45 min.

Amplification

The amplification of the RT products was assessed by using the polymerase chain reaction (PCR) technique.[3,4] Amplification was performed in two different tubes: One for SMN and the other for HUMEF1AB. Four synthesized oligonucleotides (c) to (f) (Genosys) were used. They have the following sequences: 5′CCAGGTCTAAAATTCAATGG3′ (c) for the forward primer of SMN, 5′CTGTCTGATCGTTTCTTTAG3′ (d) for the reverse primer of SMN, 5′TGTATTGGATTGCCACACG3′ (e) for the forward primer of HUMEF1AB and 5′CTTCAGCTCAGCAAACTTG3′ (f) for the reverse primer of HUMEF1AB. The oligonucleotides (c) and (e) (forward primers) were based on the SMN and HUMEF1AB sequences between base pairs 661–680 and 672–690, respectively. The oligonucleotides (d) and (f) (reverse primers) were based on the SMN and HUMEF1AB sequences between base pairs 957–976 and 705–723, respectively, in this case, however, taking the complementary sequence to allow PCR. Amplification was conducted using a DNA Thermal Cycler (Amplitron® II Thermolyne). The reaction was conducted in a total volume of 75 µL with 2 U of Taq DNA polymerase (Promega Corporation, Madison, WI, USA) in the presence of the PCR reaction buffer from Promega kit containing 0.3 nmol each of oligonucleotides, 15 pmol each of nucleotides dATP, dCTP, dGTP, and dTTP, 94 pmol of $MgCl_2$ (Promega) and 15 µL of the reverse transcription reaction medium obtained previously. Amplification conditions were as follows: Denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and elongation at 72°C for 1 min, each for 25 cycles. The PCR products were analyzed, unless otherwise noted, by electrophoresis on a 20 g L^{-1} agarose gel to screen for the presence of the appropriate-size band using the fluorescent dye ethidium bromide. Amplifying the RT products by the PCR technique[3,4] was also performed in the presence of 0.75 pmol of digoxi-

genin-11-dUTP (Boehringer Mannheim, GmbH, Germany). The same conditions for PCR were used as described previously. The PCR products were also analyzed by electrophoresis on a $20\,g\,L^{-1}$ agarose gel. The labeling of nucleic acids with digoxigenin was visualized by transfer of the DNA fragments to $40\,cm^2$ of the nitrocellulose membrane according to the transfer technique described by Southern.[21] The nitrocellulose membrane was then blocked in $12\,mL\,cm^{-2}$ of blocking solution (2% bovine serum albumine, BSA, in phosphate-buffered saline, PBS). After incubation for 1 h at 37°C, the nitrocellulose membrane was washed with PBS and then incubated for 1 h at 37°C in $12\,mL\,cm^{-2}$ of blocking solution containing 0.1% Tween® 20 and 3 μL of anti-digoxigenin antibody from sheep, conjugated with alkaline phosphatase (Boehringer Mannheim, GmbH, Germany). Then, the nitrocellulose membrane was washed with PBS and alkaline phosphatase activity was measured in the presence of chemiluminescent substrate: Disodium-2-chloro-5-(4-methoxyspiro-{1,2-dioxetane-3,2′-(5′-chloro)tricyclo [3.3.1.1.] decan}-4-yl)-1-phenyl phosphate (CDP-Star™; Boehringer Mannheim, GmbH, Germany). Reaction conditions for CDP-Star™ were as follow: $1 \times 10^{-1}\,mol\,L^{-1}$ *Tris*-HCl, pH 9.5, $1 \times 10^{-1}\,mol\,L^{-1}$ NaCl, $5 \times 10^{-2}\,mol\,L^{-1}$ $MgCl_2$, $250\,nmol\,mL^{-1}$ CDP-Star™. After incubation for 5 min at room temperature, autoradiography was developed using the BIOMAX™MR emulsion film (Eastman Kodak Co. Rochester, NY 14650, USA).

Construction of the Labeled Nucleotide Probes

Both exons 7 and 8 of the SMN gene were checked for the SMA diagnosis. The HUMEF1AB gene was used as internal standard for the control of the RT-PCR reactions.

The RT products were first amplified by the PCR technique performed in the same conditions as described previously using the synthesized oligonucleotides (e) and (f) for HUMEF1AB gene and the synthesized oligonucleotides (d), (g), (h) and (i) for SMN gene. They have the following sequences: 5′GTTTCAGACAAAATCAAAAAG3′ (g) (forward primer), 5′TCCTTAATTTAAGGAATGTGA3′ (h) (reverse primer), 5′GAAATGCTGGCATAGAGCAG3′ (i) (forward primer). The oligonucleotides (g) and (i) (forward primers) were based on exons 7 and 8 of the SMN sequences between base pairs 869–889 and 922–941, respectively. The SMN oligonucleotide (h) (reverse primer) was based on exon 7 of the SMN sequence between base pairs 901 and 921, in this case however, taking the complementary sequence to allow PCR. The PCR products were then analyzed by ethidium bromide-stained agarose gel, isolated, and purified by phenol–chloroform extraction, dried and resuspended in distilled water

according to the method described by Sambrook.[1] The purified PCR products so obtained were then polished with Pfu DNA polymerase (Stratagene). The reaction was conducted in the presence of the reagents for PCR polishing of the Stratagene kit and in a total volume of 10 μL containing 2.5 nmol each of nucleotides dATP, dCTP, dGTP, and dTTP, 2.5 U of Pfu polymerase. Reaction conditions (72°C for 30 min) in accordance with the manufacturer's recommendations were used. The blunt-ended PCR products were then subjected to the ligation reaction into the Bluescript KS(+) plasmid vector predigested by EcoRV (Gibco BRL®). The reaction was conducted in the presence of the reagents for the ligation of the Boehringer Mannheim kit (Rapid DNA ligation kit, Boehringer Mannheim, GmbH, Germany), and in a total volume of 20 μL containing 0.1 μg of digested Bluescript plasmid vector, 1.6 ng of insert DNA, 5 U T4 DNA ligase. The reaction conditions (5 min at room temperature) used were as described by the manufacturer's recommendations. After purification by phenol–chloroform extraction, the ligation products were introduced in E.Coli SURE strain by electroporation. The screening for inserts was performed using blue–white color selection. The nucleotide probes so obtained (probes 1, 2 and 3 directed at exons 7 and 8 of the SMN and HUMEF1AB genes, respectively) were then labeled with 1×10^{-3} nmol of α-^{32}P-dCTP (Amersham International) using the previous synthesized oligonucleotides (d, g, h, i for the probes 1 and 2 and e, f for the probe 3) and the reagents (dATP, dGTP, dTTP, Klenow enzyme) of the Random Primed DNA Labeling Kit (Boehringer Mannheim, GmbH, Germany). The standard assay conditions found in the manufacturer's recommendations for this kit were used. The same conditions of labeling were used for the labeling of these three probes 1, 2 and 3 with biotin-11-dCTP (Sigma, St Louis, MO). The labeling of nucleotide probes with biotin was visualized using the same conditions as that used for visualization of digoxigenin-labeled nucleic acids. Here, 15 μL of anti-biotin-monoclonal antibody conjugated with alkaline phosphatase (Boehringer Mannheim, GmbH, Germany) in 12 mL cm^{-2} of blocking solution containing 0.1% (v/v) Tween® 20 were used.

Use of Radioactive Label for Measurement of mRNA

The total RNA isolated from negative (control) and positive SMA samples (giving a negative and positive results, respectively, in DNA molecular diagnosis of SMA by means of the SSCP technique) were subjected to RT-PCR (in the absence of digoxigenin-11-dUTP) and analyzed by polyacrylamide gel electrophoresis (50 g L^{-1} acrylamide, 0.5 g L^{-1} bisacrylamide). Following the transfer of the gel to a nylon membrane of 40 cm^2

(Nylon Hybond TM-N, Amersham International), the dotted nucleic acids were UV cross-linked to the nylon membrane and hybridized with 12 mL of the hybridization solution ($5 \times$ standard saline citrate, SSC, 50% formamide, $50 \times$ Denhardt) containing 18 µL of ^{32}P-dCTP labeled nucleotide probe. Hybridization was performed overnight at 42°C under stirring. After hybridization, the membrane was washed for 10 min at 42°C in 20 mL of $5 \times$ SSC, 10 g L^{-1} SDS and for 30 min at 50°C in 20 mL of $2 \times$ SSC, 10 g L^{-1} SDS. This washing was followed by a stringent wash for 30 min at 50°C in 20 mL of $0.5 \times$ SSC, 10 g L^{-1} SDS. The membrane was then directly used for the detection of hybridized probe by autoradiography as described above and quantified by means of Bio-Imager (Fuji).

Use of Biotin Label in ELISA Procedure for Measurement of mRNA

Polystyrene microtitration plates (Maxisorb 96, Immuno Plate, Nunc) were used as the solid phase for the assays. All washes were performed four times with PBS. The substrate solutions for peroxidase, containing 5×10^{-3} mol L^{-1} tetramethyl benzidine (TMB) (Sigma, St Louis, MO) and 2.5×10^{-2} mol L^{-1} H_2O_2, was prepared in citrate phosphate buffer (1×10^{-1} mol L^{-1}; pH 5.5). After incubation for 15 min at 37°C, the reaction was stopped by the addition of 0.1 mL of 5×10^{-1} mol L^{-1} H_2SO_4. The optical density at 450 nm (OD_{450}) was measured in a microplate colorimeter (Metertech 960).

For the assay, wells of the microtitration plates were coated with streptavidin (Sigma, St. Louis, MO) (1 µg per well) in sodium carbonate buffer (1×10^{-1} mol L^{-1}; pH 9.6). After incubation overnight at 4°C, the plates were washed, and the uncoated attachment sites on the plates were saturated by incubation for 1 h at 37°C with a solution of BSA (10 g L^{-1}) and salmon sperm DNA (100 µg mL^{-1}) in PBS. The plates were then washed and the coated plates so obtained are ready to use.

For measurement of mRNA, the mRNA isolated from negative (control) and positive SMA samples were first subjected to RT-PCR in the presence of digoxigenin-dUTP as described above. An aliquot of 30 µL of each PCR product was removed and added to a mixture composed of 15 µL of hybridization solution containing salmon sperm DNA 100 µg mL^{-1} and 12 µL of biotin-labeled nucleotide probes 1, 2, or 3. After denaturation at 97°C for 10 min, hybridization was performed for 1 h at 42°C. After hybridization, 55 µL of the reaction medium was removed and added to the coated plates. After incubation for 1 h at 37°C, the plates were washed, and 100 µL of a 1-in 1,000 dilution of horseradish peroxidase-labeled sheep antibody

anti-digoxigenin (Boehringer Mannheim, GmbH, Germany) in PBS containing 0.05% (v/v) Tween® 20 was added. After being incubated again for 1 h at 37°C, the plates were washed, and substrate solution was added.

RESULTS AND DISCUSSION

To develop a quantitative method for molecular diagnosis of genetic disorders, we have used both radioactive nucleotide probes and the non-radioactive ELISA method to measure the amount of cytosolic mRNA from human muscle cells.

As shown in Tables 1 and 2, there was a perfect concordance of results obtained between the procedure using radioactive material, the ELISA non-radioactive method and the SSCP analysis regarding the negative and positive SMA samples. All values obtained for the control group were significantly greater than the ones obtained for the SMA positive samples (33–76% in the radioactive method and 38–54% in the ELISA method). Lefebvre et al.,[16] reported that 93% of SMA patients lacked the SMN exons 7 and 8 on both mutant chromosomes, for all three types of SMA. These authors also reported that the SMN transcripts were absent and that the C_{BCD541} transcripts were solely present in patients lacking the SMN gene on both mutant chromosomes, while control individuals expressed both RNA transcripts. They also revealed that the centromeric transcripts, but not the SMN gene transcripts, may normally undergo alternative splicing of exon 7 to produce transcripts lacking this exon. Analysis of a control population showed that the SMN gene was present in all individuals while the C_{BCD541} gene was present in 95.5%.[16] Taking into account the above mentioned T_{BCD541} and C_{BCD541} problem, the quantitative method based on the measurement of specific mRNA may be useful for the molecular diagnosis of SMA. Despite the small number of samples examined (5 negative and 13 positive SMA samples), the results of this study demonstrate that the measurement of mRNA could be used as a quantitative method for the molecular diagnosis of SMA.

Current techniques are available for the analysis of mRNA, however, because each assay has its own problem, no assay has been accepted as routine for diagnostic purposes. For example, Northern blotting[1] is labor intensive and is not suitable for quantification of mRNA because of uncertainty as to which fraction of applied mRNA is immobilized on the membranes, and most importantly, because some regions of mRNA may be used for immobilization rather than hybridization. RNase protection assay[2] is more sensitive than Northern blotting but usually requires radioactive material and labor-intensive steps, which may not be suitable for assaying

Table 1. Comparison of Diagnostic Methods for SMA

	SSCP[1]	Our Method (Radioactive)[3]					
		Probe 1 (Exon 7)			Probe 2 (Exon 8)		
N° Tube	Exons[2]	PSL/mm²	R (%)[4]	Exon 7	PSL/mm²	R (%)[4]	Exon 8
Control (1)	ndel 7/ndel 8	42	0	ndel	22	0	ndel
Control (2)	ndel 7/ndel 8	41	0	ndel	23	0	ndel
Control (3)	ndel 7/ndel 8	43	0	ndel	22	0	ndel
Control (4)	ndel 7/ndel 8	41	0	ndel	21	0	ndel
Control (5)	ndel 7/ndel 8	42	0	ndel	23	0	ndel
SMA (6)	del 7/del 8	24	43	del	15	32	del
SMA (7)	del 7/del 8	14	67	del	08	64	del
SMA (8)	del 7/del 8	10	76	del	07	68	del
SMA (9)	del 7/del 8	26	38	del	06	73	del
SMA (10)	del 7/del 8	09	79	del	13	41	del
SMA (11)	del 7/del 8	27	36	del	12	45	del
SMA (12)	del 7/del 8	15	64	del	15	32	del
SMA (13)	del 7/del 8	13	69	del	14	36	del
SMA (14)	del 7/del 8	25	40	del	11	50	del
SMA (15)	del 7/del 8	20	52	del	09	59	del
SMA (16)	del 7/del 8	19	55	del	13	41	del
SMA (17)	del 7/del 8	12	71	del	14	36	del
SMA (18)	del 7/del 8	14	67	del	12	45	del

[1]Single strand conformation polymorphism. [2]del: deleted; ndel: non deleted. [3]The quantification of results obtained is performed by means of Bio-Imager and expressed as PSL/mm². [4]R: difference = 1 − [(PSL/mm²Control−PSL/mm²SMA)/(PSL/mm²Control)]. The mean value of the control group is used for the calculation of R.

Table 2. Comparison of Diagnostic Methods for SMA

	SSCP[1]	Our Method (ELISA)							
		Probe 1 (Exon 7)			Probe 2 (Exon 8)			Probe 3 (HUMEF1AB)	
N° tube	Exons[2]	Optical Density	R (%)[3]	Exon 7	Optical Density	R (%)[3]	Exon 8	Optical Density	RT-PCR
Control (1)	ndel 7/ndel 8	0.26	0	ndel	0.28	0	ndel	0.55	positive
Control (2)	ndel 7/ndel 8	0.28	0	ndel	0.29	0	ndel	0.54	positive
Control (3)	ndel 7/ndel 8	0.27	0	ndel	0.26	0	ndel	0.52	positive
Control (4)	ndel 7/ndel 8	0.26	0	ndel	0.28	0	ndel	0.51	positive
Control (5)	ndel 7/ndel 8	0.27	0	ndel	0.27	0	ndel	0.49	positive
SMA (6)	del 7/del 8	0.16	41	del	0.13	54	del	0.51	positive
SMA (7)	del 7/del 8	0.16	41	del	0.16	43	del	0.48	positive
SMA (8)	del 7/del 8	0.11	59	del	0.12	57	del	0.53	positive
SMA (9)	del 7/del 8	0.15	44	del	0.14	50	del	0.49	positive
SMA (10)	del 7/del 8	0.19	30	del	0.16	43	del	0.5	positive
SMA (11)	del 7/del 8	0.13	52	del	0.12	57	del	0.49	positive
SMA (12)	del 7/del 8	0.14	48	del	0.13	54	del	0.48	positive
SMA (13)	del 7/del 8	0.17	37	del	0.14	50	del	0.55	positive
SMA (14)	del 7/del 8	0.12	55	del	0.15	46	del	0.52	positive
SMA (15)	del 7/del 8	0.11	59	del	0.13	54	del	0.5	positive
SMA (16)	del 7/del 8	0.13	52	del	0.15	46	del	0.51	positive
SMA (17)	del 7/del 8	0.15	44	del	0.12	57	del	0.49	positive
SMA (18)	del 7/del 8	0.16	41	del	0.16	43	del	0.5	positive

[1]Single strand conformation polymorphism. [2]del: deleted; ndel: non deleted. [3]R: difference = 1 − [(OD_{450}Control − OD_{450}SMA/OD_{450}Control)]. The mean value of the control group is used for the calculation of R.

large numbers of clinical specimens. PCR[3,4] and other gene amplification procedures may give problems in quantification and reproducibility, although such assays provide the best sensitivity. In situ hybridization[5] and in situ PCR[6] are the only available techniques for localization of gene expression; however each specimen must be examined microscopically by expert pathologists with expensive imaging equipment for quantification.

As a result of this study, based on the measurement of specific mRNA, both the procedure using radioactive material and the ELISA non-radioactive method could be applicable to the molecular diagnosis of all genetic disorders associated with a deletion or mutation of gene(s), such as Duchene myopathies, mucoviscidose, or genetic disorders associated to a duplication of the gene such as Charcot—Marie—Tooth disease type 1A. Here, it is important to note that besides the hazardous problem related to the use of a mutagenic compound (ethidium bromide) for analysis of the PCR results, the qualitative techniques using the SSCP technique[16] and the restriction enzymes[17] for the molecular diagnosis of SMA at the DNA level do not allow the detection of heterozygous deletion in exons 7 and 8 of the SMN gene (SMA carriers). To overcome this problem, using the mRNA titration curve, our quantitative methods based on the measurement of mRNA may be useful. Moreover, the methods developed in this study may also be useful in the control of mRNA concentrations in medicine and in gene therapy. Indeed, the concentrations of each specific mRNA are different in normal and in disease states and they also change rapidly in response to various clinical treatments.

CONCLUSION

The procedure using radioactive material and the ELISA non-radioactive method could both be applicable to the molecular diagnosis of SMA. However, in order to be widely used in clinical laboratories, the diagnostic technique must be safe, easy to handle and automated. For such a purpose, the ELISA method appears to be the best technique because it does not use radioactive material. Furthermore, in comparison to the expensive equipment such as the fluorescent and chemiluminescent plate readers, the colorimetric ELISA readers are less expensive and are widely available in any laboratory. Our ELISA method requires minimal time for setup (only 3 h to complete the test), and it is easy to interpret the quantitative results obtained. In addition, the precoated plates and the biotinylated nucleotide probes can be prepared in advance and stored without a decrease in reactivity.

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